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Crystallization and preliminary X-ray analysis of CTLA-4 (CD152) membrane-external domain

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CTLA-4 (CD152) is involved in T-lymphocyte co-stimulatory pathways modulating both humoral and cellular immune response. The membrane-external domain has been prepared and crystallized. The unit-cell parameters are $a = b = 43$, $c = 143$ Å with the symmetry of space group $P3_121$ or its enantiomer and the crystals diffract to 2.7 Å resolution at synchrotron beamlines.

1. Introduction

In order for T lymphocytes to successfully initiate cell-mediated and/or antibody-mediated immune responses to an antigenic stimulus, distinct activation signals are required from antigen-presenting cells (activated B cells, dendritic cells, monocytes/macrophages) (Bretscher, 1992). An antigen-specific signal occurs when particular T-cell antigen receptors bind to antigenic peptides presented by major histocompatibility complex (MHC) molecules (class I or II) on the surface of antigen-presenting cells. This event alone is not sufficient to stimulate a productive immune response and by itself can lead to T-cell inactivation or clonal unresponsiveness (anergy) (Schwartz, 1990). For a full immune response to occur, T-cells must receive a second, antigen non-specific, costimulatory signal mediated through other receptor/counter-receptor interactions with antigen-presenting cells (Linsley & Ledbetter, 1993).

An important costimulatory pathway which is critical in regulating immune responses to antigens is the interaction between the CD28 receptor on T-lymphocytes and its counter receptors, CD80 (B7-1) and CD86 (B7-2), on antigen-presenting cells (Linsley & Ledbetter, 1993; June *et al.*, 1994). The functional outcome of these interactions is the clonal proliferation of antigen-reactive lymphocytes and production of T-cell derived cytokines that profoundly influence both cell-mediated and humoral immune responses. CTLA-4 (CD152) is a T-cell surface molecule whose expression is up-regulated upon T-cell activation. It is homologous to CD28; both receptors belong to the immunoglobulin supergene family by virtue of their extracellular regions containing a single immunoglobulin domain similar to the immunoglobulin variable domain (V-like; nine-stranded antiparallel β-barrel). CTLA-4 also binds to CD80 and CD86, but with >500-fold higher avidity than CD28 (Greene *et al.*, 1996). CD80 and CD86 are also homologous members of the immunoglobulin supergene family, with both molecules having extracellular domains containing an N-terminal immunoglobulin V-like domain and a membrane-proximal immunoglobulin C-like domain (seven-stranded antiparallel β-barrel). CTLA-4 and CD28 are disulfide-linked dimers with the interchain disulfide cysteine occurring in the membrane-proximal region. In the absence of this disulfide bond, the extracellular V-like domain has been shown by structural studies to be monomeric in solution (Metzler *et al.*, 1997).

CTLA-4 is thought to deliver signals to T-cells that down-regulate their activity, thus countering the co-stimulatory properties of CD28 (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). A soluble form of the CTLA-4 receptor, CTLA4Ig, has been prepared by fusing the V-like extracellular domain of CTLA-4 to an IgG1 Fc. CTLA4Ig has immunosuppressive properties. It acts by binding to CD80 and CD86, thereby preventing their interaction with CD28 and thus blocking T-cell activation and effector functions. In animal models, CTLA4Ig has been shown to inhibit T-cell-dependent antibody responses, significantly prolong transplanted organ survival, induce long-term donor-specific tolerance and slow progression of autoimmune diseases (Peach & Linsley, 1995).

In an effort to aid design of higher affinity variants of CTLA4Ig, we set out to determine the structure of the membrane-external V-like domain of CTLA-4. In this paper, we describe the preparation, crystallization and preliminary X-ray analysis of CTLA-4 membrane-external domain.

2. Materials and methods

Partially deglycosylated CTLA4-Ig was prepared essentially as described by Metzler *et al.* (1997). Briefly, CTLA4Ig was purified from the culture media of Chinese hamster ovary

Table 1
Summary of synchrotron data sets.

	SSRL 7-1		CHESS F1	
	Overall	Last shell	Overall	Last shell
Resolution (Å)	40–2.6	2.69–2.6	40–2.8	2.9–2.8
No. measured reflections	19557	≥1674	14215	584
No. unique reflections	4921	481	3783	327
Completeness (%)	95	94	85	78
R_{sym}	0.041	0.463	0.051	0.340
$I/\sigma(I)$	27.9	2.8	24.3	3.2

(CHO) cells by protein A chromatography (Pharmacia), treated with neuraminidase (New England Biolabs) and treated with thrombin (Linsley *et al.*, 1995) to cleave the fusion protein between the CTLA4 extracellular domain and the IgG1 Fc. To obtain monomer, the cleaved dimer was mildly reduced with 1 m*M* dithiothreitol and the free sulfydryls alkylated with iodoacetamide. The monomer, CTLA4ex, was purified by ion-exchange chromatography on a Resource-Q (Pharmacia) column. The carbohydrates were trimmed by treatment with *Arthobactor ureafaciens* sialidase, *Streptococcus pneumoniae* β-galactosidase, jack-bean β-N-acetylhexosaminidase and jack-bean α-mannosidase (Oxford Glycosystems) in the buffer supplied with the α-mannosidase (100 m*M* sodium acetate, 2 m*M* $ZnCl_2$ pH 5). The CTLA4ex was further fractionated on a Sephacryl S-200 column (Pharmacia) in 20 m*M* Tris–HCl pH 7.4, 100 m*M* NaCl, 1 m*M* EDTA to remove the enzymes. Finally, the protein was concentrated and buffer-exchanged into 10 m*M* HEPES pH 7.2, 0.1% azide at a final protein concentration of 1.3–4.5 mg ml^{-1} in an Ultrafree centrifugal ultrafilter (Millipore).

Crystallization trials were performed using the sitting-drop vapor-diffusion method (McPherson, 1982, 1990) with a droplet consisting of 1 µl of protein solution and 1 µl precipitant reservoir solution on Cryschem plates (C. Supper Co., Natick, MA, USA). Reservoir solutions were initially screened using complete reagent kits (Crystal Screens I and II, Grid Screen AS, PEG 6K, PEG 6K/LiCl, NaCl, MPD *etc.*, all from Hampton Research, California, USA). All experiments were carried out at 295 K.

Optimization of promising trials from Crystal Screens was carried out by varying the pH, organic additives and concentrations of the components of the mother liquor as described elsewhere (McPherson, 1990). Disc-shaped or octahedral-shaped CTLA4ex crystals were obtained at ~295 K using polyethylene glycol (PEG) 3350 as a precipitant. The hanging drops contained 5 µl of CTLA4ex and 4 µl of a reservoir solution consisting of 50 m*M* sodium cacodylate pH 6.5, 20–25% PEG 3350, 100 m*M* ammonium acetate, 3% acetone. Drops were pre-equilibrated in Linbro tissue-culture plates against 1 ml of reservoir solution before macroseeding in order to obtain diffraction-size crystals.

3. Results and discussion

For crystallographic characterization, a small crystal was mounted in a glass capillary. Initial data could be indexed in a trigonal cell of unit-cell parameters $a = b = 43$, $c = 143$ Å with the symmetry of space group $P3_121$ or its enantiomer and with one monomer per asymmetric unit. The molecules are rather loosely packed, with a Matthews volume (Matthews, 1968) V_M of 3.1 Å^3 Da^{-1} and a solvent content of 61%.

Finding stabilizing conditions for flash-cooling CTLA4ex crystals proved extremely difficult because most cryoprotectant solutions cracked crystals, destroyed diffraction or yielded crystals with too large a mosaic spread. A procedure was finally found that increased xylitol concentrations stepwise to 30%(*w*/*v*) and satisfactorily preserved the crystalline condition, *i.e.* mosaic spreads of 0.6–0.8°. A data set to 3.3 Å resolution was collected in the laboratory and data sets to 2.6 and 2.8 Å resolution were collected at SSRL beamline 7-1 using a MAR 30 cm detector and at CHESS F1 using the Princeton 2K CCD detector, respectively (Tate *et al.*, 1995; Thiel *et al.*, 1996). The data were processed with *HKL* (Otwinowski & Minor, 1997) and statistics are summarized in Table 1 for the two synchrotron data sets. In the data collected at synchrotrons a marked fall-off was noticed with resolution. The isotropic *B* factor of the data was estimated using the program *ANISOB* (Sheriff & Hendrickson, 1987) to be 47 and 56 Å^2, respectively. This high *B* factor is consistent with the loose packing predicted by the Matthews volume and solvent content.

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